THE GENTAMICIN ANTIBIOTICS. 6[1)]

GENTAMICIN C_{2b}, AN AMINOGLYCOSIDE ANTIBIOTIC PRODUCED BY *MICROMONOSPORA PURPUREA* MUTANT JI-33

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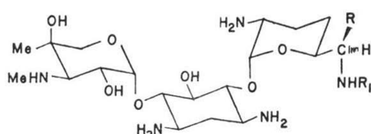
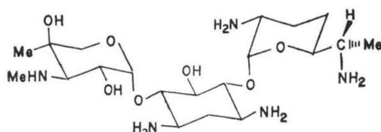
A mutant strain of *Micromonospora purpurea*, designated var. JI-33, produced an antibiotic complex consisting primarily of gentamicin C_{1a}. A further product of this fermentation was identical to a very minor component isolated from the fermentation of the parent organism and named gentamicin C_{2b}. Physical measurements indicated its structure to be 6′-*N*-methylgentamicin C_{1a} and this was confirmed by synthesis from gentamicin C_{1a}. The *in vitro* antibacterial activity of gentamicin C_{2b} was very similar to that of the gentamicin C complex. Antibiotic XK-62-2, produced by *Micromonospora sagamiensis*, appears to be identical to gentamicin C_{2b}.

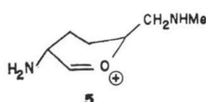
Fermentation of *Micromonospora purpurea* NRRL 2953 affords, as the major products, the important antibiotics gentamicins C_1, C_2 and C_{1a} (gentamicin C complex)[2)] as well as a host of minor components.[3)] During the screening of this organism, in search of improved gentamicin producers, a novel variant, designated *M. purpurea* var. JI-33, was isolated. The strain, obtained by multiple nitrosoguanidine treatments and repeated strain selection, was found to possess similar morphology to that already described for gentamicin-producing strains of *M. purpurea*.[4)] The new strain, JI-33, however, produced an aminoglycoside antibiotic complex different from the normal gentamicin fermentations. The major product isolated from the JI-33 fermentation broth proved to be gentamicin C_{1a} (**1**);[1,5,6)] gentamicins C_2 (**2**) and C_1 (**3**) were not observed. A minor but substantial constituent of the fermentation, migrating similarly to gentamicin C_2 on chromatography, was isolated and designated antibiotic JI-33B. Smaller amounts of more polar components were also observed.



1	Gentamicin C_{1a}	$R = R_1 = H$
2	Gentamicin C_2	$R = Me$; $R_1 = H$
3	Gentamicin C_1	$R = R_1 = Me$
6	Gentamicin C_{2b}	$R = H$; $R_1 = Me$



4 Gentamicin C_{2a}



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Several years ago a very minor component of the gentamicin fermentation was isolated in our laboratories using countercurrent distribution and was named gentamicin C_{2a}. Evidence suggested that this compound was

the 6′-epimer (**4**) of gentamicin C_2.[7] More recently a further, very minor component was isolated and named gentamicin C_{2b}. Chromatographic, spectral and microbiological comparisons revealed that antibiotics JI-33B and gentamicin C_{2b} were identical. The name gentamicin C_{2b} has been adopted for this antibiotic in view of its structural similarity to the other gentamicin C components. The chromatographic mobilities of gentamicins C_1, C_2, C_{2b} and C_{1a} in several systems are shown in Table 1. A paper chromatographic system[8] (Table 1, system 4) was found which differentiated all the gentamicin C components.

Gentamicin C_{2b} was obtained as a white amorphous solid; its molecular weight by mass spectrometry was 463, the same as gentamicin C_2. Principal fragments in its mass spectrum indicated the presence of garosamine, 2-deoxystreptamine and a third sugar giving rise to a prominent ion at *m/e* 143 (**5**)[9] (see Experimental section). A characteristic feature of the mass spectrum was a relatively intense peak at *m/e* 433; no analogous peak was observed in the mass spectrum of gentamicin C_{1a} (**1**). This peak is thought to arise by the loss of a CH_4N fragment from the molecular ion or a CH_3NH_2 fragment from the MH^+ ion. The pmr spectrum of gentamicin C_{2b} base is shown in Fig. 1A, and of the acidified solution in Fig. 1B.

Table 1. Chromatographic mobilities of gentamicins C_1, C_2, C_{2b}, C_{1a} and antibiotic XK-62-2[a)]

Antibiotic	Chromatographic system[b)]			
	R_F^1	R_F^2	R_F^3	$R_{C_1}^{4\ c)}$
Gentamicin C_1	0.53	0.64	0.28	1.00
Gentamicin C_2	0.42	0.63	0.22	0.56
Gentamicin C_{2b}	0.40	0.55	0.23	0.73
Gentamicin C_{1a}	0.33	0.52	0.15	0.29
Antibiotic XK-62-2	0.40	0.55		0.73

a) Systems 1, 2 and 3 were thin-layer chromatograms performed on ANALTECH plates (Newark, Delaware) coated with silica gel GF (250 μ). Plates were activated by heating for 1 hour at 100°C prior to use. System 4 was a paper chromatograph performed on Whatman #1 paper; chromatograms were developed for 4 hours.

b) System 1: Lower phase of a 1:1:1 mixture of chloroform-methanol-15 N ammonium hydroxide as eluant. Compounds were visualized by sulfuric acid charring.
System 2: 1:2:1 mixture of chloroform-methanol-15 N ammonium hydroxide as eluant. Compounds were visualized by sulfuric acid charring.
System 3: Lower phase of a 2:1:1 mixture of chloroform-methanol-15 N ammonium hydroxide as eluant. Compounds were visualized by sulfuric acid charring.
System 4: Lower phase of a 2:1:1 mixture of chloroform - methanol - 9 N ammonium hydroxide as eluant. Compounds were visualized by overlaying the dried paper on *Staphylococcus aureus*, incubating overnight and observing the zones of bacterial inhibition.

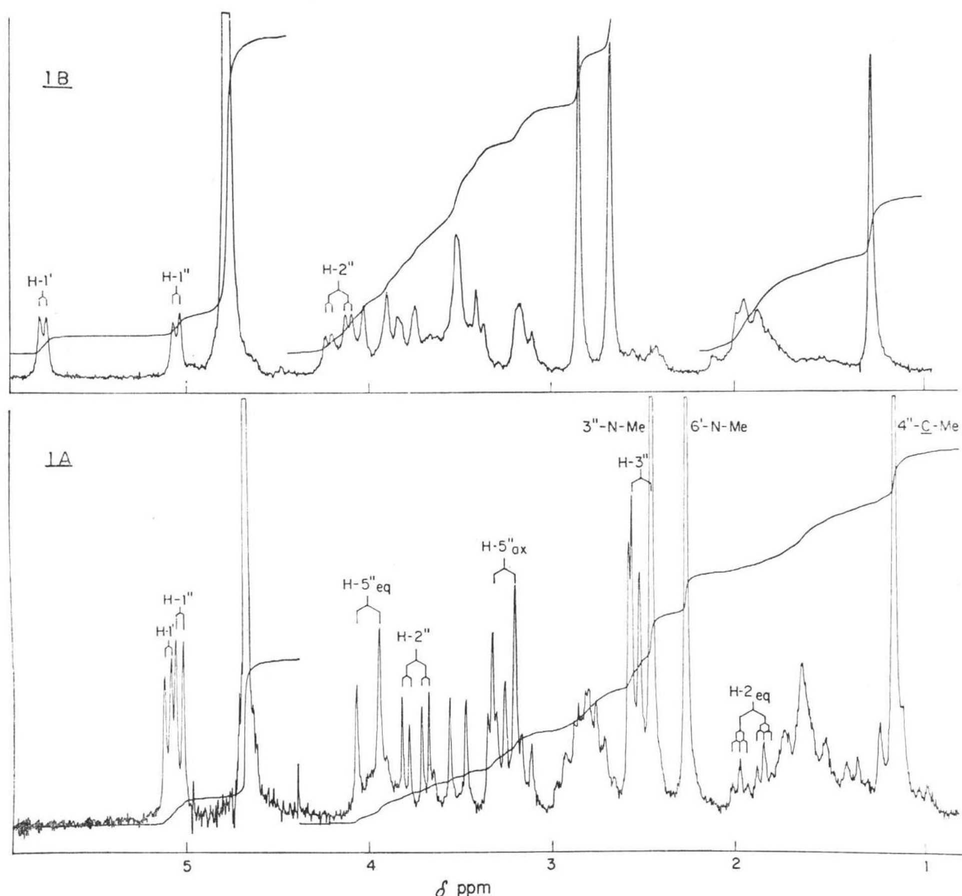
c) $R_{C_1} = \dfrac{\text{distance moved by component from origin}}{\text{distance moved by gentamicin } C_1 \text{ from origin}}$

Table 2. ^{13}C Chemical shifts[a)] of gentamicins C_{1a}, C_{2b} and antibiotic XK-62-2

Carbon	Gentamicin C_{1a} (1)[9]	Gentamicin C_{2b} (6)	Antibiotic XK-62-2
1	51.7	51.4	51.4
2	36·7	36.4	36.4
3	50.6	50.3 (50.5)	50.3 (50.5)
4	88.3	87.8 (87.4)	87.8 (87.1)
5	75.4	75.4	75.4
6	87.8	87.4 (87.8)	87.1 (87.8)
1′	102.2	101.7 (101.3)	101.4 (101.3)
2′	51.0	50.5 (50.3)	50.5 (50.3)
3′	27.1	26.5	26.4
4′	28.5	28.5	28.4
5′	71.5	68.5	68.5 (68.2)
6′	46.1	55.3	55.1
6′-*N*-Me		35.4	35.3
1″	101.3	101.3 (101.7)	101.3 (101.4)
2″	70.2	70.2	70.1
3″	64.4	64.2	64.2
4″	73.3	73.2	73.2
5″	68.7	68.5	68.2 (68.5)
3″-*N*-Me	38.0	37.6	37.6
4″-*C*-Me	23.0	22.3	22.3

a) Shifts downfield from TMS. In a number of cases resonances for **6** were too close together for unequivocal assignment. Alternate assignments are given in parentheses.

Fig. 1. A. PMR spectrum (100 MHz, D_2O) of gentamicin C_{2b} base.
B. PMR spectrum of gentamicin C_{2b} in acid.



In the spectrum of the base, resonances at δ 5.04 (1H, doublet, J=4 Hz), 4.01 (1H, doublet, J=12.5 Hz), 3.75 (1H, quartet, J=4, 11 Hz), 3.27 (1H, doublet, J=12.5 Hz), 2.52 (1H, doublet, J=11 Hz), 2.46 (3H, singlet) and 1.15 ppm (3H, singlet) were characteristic of protons 1″, $5''_{eq}$, 2″, $5''_{ax}$, 3″ and the *N*-CH_3 and *C*-CH_3 groups of the garosamine unit.[1] A doublet of triplets at δ 1.92 ppm was characteristic of H-2_{eq} of the 2-deoxystreptamine ring. The second sugar gave rise to a doublet at δ 5.11 ppm (J=3.5 Hz) due to the anomeric proton and an *N*-CH_3 singlet at δ 2.27 ppm.

In view of the structural relationships of gentamicins C_1, C_2 and C_{1a}, which differ only in the degree of methylation of the 2,6-diaminosugar (purpurosamine[5]) unit, these data suggested that gentamicin C_{2b} was 6′-*N*-methylgentamicin C_{1a} (**6**). This was supported by the cmr spectral data shown in Table 2. The assignments of resonances for gentamicin C_{1a} have been discussed previously.[10] Inspection of the data in Table 2 reveals a close agreement between the resonance values of gentamicins C_{1a} and C_{2b} with the exception of an extra peak at 35.4 ppm in the spectrum of C_{2b}, attributed to the extra *N*-CH_3 carbon atom, and the resonances for carbons 5′ and 6′ which are shifted 3.0 ppm upfield and 9.2 ppm downfield respectively in the spectrum of C_{2b}. The direction and magnitude of these shifts is consistent with the location of the extra

N-CH_3 group of C_{2b} on the 6′-nitrogen atom.

Confirmation of the structure of gentamicin C_{2b} was provided by its synthesis from gentamicin C_{1a}. Reaction of this latter compound with *t*-butoxycarbonyl azide gave the 6′-*N*-*t*-butoxycarbonyl derivative which was reduced by lithium aluminum hydride to gentamicin C_{2b} (**6**).

A material claimed to have the same structure as gentamicin C_{2b}* has been reported previously[11] as a minor component of the gentamicin complex. Structure **6** was tentatively proposed for this material, mainly on the basis of its mass spectrum, despite the fact that the pmr data given in that report[11] clearly show that the material isolated was a mixture.

The *in vitro* antibacterial activity of gentamicin C_{2b} is shown in Table 3 in comparison to that of gentamicin C complex. The M.I.C. values for the two substances are similar.

Table 3. *In vitro* antibacterial activities of gentamicin C complex and gentamicin C_{2b}

Test organism		Resistance mechanism	Minimal inhibitory concentration (mcg/ml)*	
			Gentamicin C complex	Gentamicin C_{2b}
Staphylococcus aureus	FDA 209 P		0.03	< 0.01
〃	Wood		0.03	< 0.01
Streptococcus pyogenes	C		3.0	7.5
〃	27		0.075	7.5
Escherichia coli	St.M. 589		0.75	0.75
〃	Baker 2		0.75	0.3
〃	F14-BK		0.3	0.03
〃	R5/W677	6′-*N*-acetylating	0.75	0.03
〃	LA290/R55	2″-*O*-adenylylating	17.5	7.5
〃	W677/R55	〃	17.5	>25
〃	JR88	3-*N*-acetylating	>25	>25
Pseudomonas aeruginosa	1395		0.75	0.3
〃	D-2		3.0	0.75
〃	NRRL 3223		0.3	0.03
〃	Stone 20		0.3	0.075
〃	Travers	3-*N*-acetylating	>25	>25
〃	Stone 138	〃	>25	>25
〃	Capetown 18	〃	7.5	17.5
〃	Shriners 10099	Unknown	7.5	7.5
〃	GN 315	6′-*N*-acetylating	0.3	0.03
Klebsiella pneumoniae	Ad 17		0.3	0.03
〃	Ad 18		0.3	0.075
〃	Georgetown 3694	2″-*O*-adenylylating	17.5	17.5
〃	Oklahoma	〃	>25	>25
Providence	164	2′-*N*-acetylating	17.5	25
Proteus mirabilis	Harding		3.0	0.3
Proteus rettgeri	Membel		0.3	7.5
Bacillus subtilis	6623		< 0.01	< 0.01

* In MUELLER HINTON broth, pH 7.2.

* In this publication[11], compound **6** was named gentamicin C_{2a}, a name which we had previously assigned to the compound of structure **4**.

An antibiotic named XK-62-2 was reported recently in a patent[12,14] by scientists at the Kyowa Hakko Kogyo Co., as a product of strains of *Micromonospora sagamiensis*. Gentamicins C_1, C_2 anp C_{1a} were co-produced by this organism. These workers also reported the isolation of antibiotic XK-62-2 from fermentations of *M. purpurea* and *M. echinospora*. Structure **6** was assigned to this antibiotic. We have obtained a small sample of antibiotic XK-62-2 and, on the basis of our limited findings with this sample, believe that it is identical to gentamicin C_{2b}. The pmr spectra of XK-62-2 in basic and acidic solutions are extremely similar to those of gentamicin C_{2b}. The chromatographic mobilities of the two materials are the same in three systems (Table 1) and the cmr values are identical within experimental error (Table 2). One point of apparent difference between the antibiotics is the reported rotation of antibiotic XK-62-2 (+116° in water) which is appreciably lower than the measured rotation for gentamicin C_{2b}. Unfortunately we had insufficient sample of XK-62-2 to redetermine its rotation value, however, in view of the hygroscopic nature of these antibiotics and the difficulty sometimes observed in obtaining accurate rotations for antibiotics of this type* we believe that the compounds are in fact the same in view of the otherwise identical physical parameters.

Experimental

FOURIER transform NMR spectra were taken in D_2O solution using a Varian XL-100-15 instrument equipped with a 620L computer. PMR shifts are reported downfield from sodium 2,2-dimethyl-2-silapentane sulfonate using the HOD line (δ 4.68 ppm) as an internal reference.

CMR shifts were referenced to internal dioxane and are reported downfield relative to TMS using the relationship $\delta_C^{TMS}=\delta_C^{diox}+67.4$ ppm. Mass spectra were obtained using a Varian MAT CH5 spectrometer at 70 eV with a probe temperature of 150~250°C. The direct inlet technique was used. Optical rotations were recorded in aqueous solution using a Bendix model 143 automatic polarimeter. Chromatography was carried out on silica gel (70~230 mesh, E. Merck, Darmstadt, W. Germany).

Fermentation and Isolation of Antibiotic JI-33 Complex.

Fermentation of strain JI-33 was carried out in shake-flasks and 10 liter fermentors under aerobic conditions. The inoculum was prepared in a medium consisting of: Bacto beef extract (3 g), Bacto tryptone (5 g), Bacto dextrose (1 g), soluble starch (24 g), yeast extract (5 g), calcium carbonate (1 g), Difco broth (15 g) and soft water (1 liter). A vigorously growing 48-hour culture was an excellent inoculum; best results were obtained with a 10% inoculum for the production stage. The production medium consisted of: potatoe dextrin (50 g), dextrose (5 g) calcium carbonate (1 g), soybean meal (35 g) and soft water (1 liter). The pH was adjusted to 7.2 using sodium hydroxide solution and GE-60 was added as an antifoam. After sterilization in an autoclave fermentations were carried out at 33~34°C with agitation at 400~500 rpm. The production peak (120~144 hours) was established by periodic sampling of the fermentation broth and assaying *versus Staphylococcus aureus* ATCC 6538-P according to the disc-plate assay procedure of ODEN *et al.*[13] using gentamicin as the standard. Peak titers of 800~1,400 mcg/ml

* We have experienced difficulty, in certain cases, in obtaining reproducible optical rotation values for aminoglycoside-aminocyclitol antibiotic bases. We have attributed this to (1) difficulty in completely removing water from the sample by vacuum drying and the hygroscopic nature of the materials which makes accurate weighings difficult; (2) problems due to carbonation of the samples with atmospheric CO_2. As an example, chromatographically homogeneous gentamicin C_{2b}, after normal isolation and drying has given rotations of +145°→148°. However, a sample which was carefully decarbonated by passage through a strongly basic ion exchange resin, collecting only the first part of the eluate, with subsequent handling under nitrogen, gave an optical rotation of +165° (average of two determinations) (see Experimental section).

were obtained.

At harvest the whole broth was acidified to pH 2 with 12 N sulfuric acid and the mycelium was removed by filtration. The filtrate was neutralized with concentrated ammonium hydroxide and calcium ions were removed by the addition of a saturated solution of oxalic acid followed by filtration of the precipitated calcium oxalate. After reneutralization the filtrate was passed through an Amberlite® IRC-72 resin (NH_4^+ cycle), the resin was washed with distilled water and the antibiotic complex eluted with 2 N ammonium hydroxide. The eluate we concentrated to low volume *in vacuo* and decolorized by passage through an Amberlite® IRA-458 resin followed by elution with distilled water. The eluate was concentrated *in vacuo* and lyophilized to give crude antibiotic JI-33 complex (yield 300~600 mcg/ml, with potency of 500~700 mcg/mg *vs* gentamicin).

Purification of Antibiotics from JI-33 Complex.

The crude antibiotic complex was chromatographed over silica gel using the lower phase of a 2:1:1 mixture of chloroform-isopropanol-concentrated ammonium hydroxide. Fractions were monitored by thin-layer chromatography. Gentamicin C_{2b} (**6**) was eluted first and comprised 5~10 % by weight of the crude antibiotic complex. Further elution gave gentamicin C_{1a} (**1**), comprising 25~50 % by weight of the crude antibiotic complex. Gentamicin C_{1a} was identified by chromatographic, spectral and microbiological comparisons with an authentic sample.

Gentamicin C_{2b}.

Final purification of this material was effected by rechromatography of a sample (1.2 g) over 120 g of silica gel, eluting with the lower phase of a 2:1:1 chloroform-methanol-10 N ammonium hydroxide solvent system. Fractions 108~124 were combined and evaporated to give gentamicin C_{2b} (**6**) (190 mg), $[\alpha]_D+145°$ (*c*, 0.3, H_2O). A sample was decarbonated by passage of an aqueous solution through a column of Amberlite IR-401S (OH^- form). The first, most basic eluate, was collected under nitrogen, lyophilized and dried at 50°C/0.1 mm for 48 hours. This sample had $[\alpha]_D^{26}+167.8°$ (*c*, 0.45, H_2O), +163.0 (*c*, 0.42, H_2O). Mass spectral peaks at *m/e* 464 $(M+1)^+$, 463 (M^+), 433 $(M-\dot{C}H_4N)$, 388 $(M-HOCH_2\dot{C}(OH)Me)$, 350, 332, 322, 304 (garosamine-2-deoxystreptamine), 333, 315, 305, 287 (2,6-diaminosugar-2-deoxystreptamine), 191, 173, 163, 145 (2-deoxystreptamine), 160, 142, 118 (garosamine), 143 (ion **5**). The pmr spectrum is shown in Fig. 1 and the cmr resonances in Table 2.

Anal. Calcd. for $C_{20}H_{41}N_5O_7 \cdot 0.5\ H_2O$: C, 50.83; H, 8.96; N, 14.82.
Found: C, 50.54; H, 9.43; N, 14.60.

6′-*N*-*t*-Butoxycarbonylgentamicin C_{1a}.

To a stirred solution of gentamicin C_{1a} (2.69 g, 6 mmole) in 50 % aqueous methanol containing triethylamine (1.82 ml), *t*-butoxycarbonyl azide (1.91 g, 13.4 mmole) was added with cooling to 5°C. After stirring for 18 hours at 5°C Amberlite IRA-401S ion-exchange resin (OH^- cycle) was added and stirring continued for a further 30 minutes. The resin was removed by filtration and the filtrate concentrated to dryness *in vacuo*. The crude product was chromatographed over silica gel using the lower phase of system 3 (Table 1) as eluant. Fractions were monitored by TLC and those containing the pure major product were pooled and lyophilized to give 6′-*N*-*t*-butoxycarbonylgentamicin C_{1a} (0.42 g, 13 %), $[\alpha]_D^{26}+137°$ (*c*, 0.3, MeOH); pmr δ 1.23 (3H, s, C-CH_3), 1.45 (9H, s, $C(C\underline{H}_3)_3$), 2.53 (3H, s, N-$C\underline{H}_3$), 5.08 ppm (2H, overlapping doublets, $J\simeq3.5$ Hz); mass spectrum *m/e* 550 $(MH)^+$, 549 M^+, 419, 401, 391, 373 (6′-*N*-*t*-butoxycarbonylgentamine C_{1a}), 350, 332, 322, 304 (garosamine-2-deoxystreptamine), 191, 173, 163, 145 (2-deoxystreptamine), 229 (6′-*N*-*t*-butoxycarbonyl-purpurosamine C), 160 (garosamine).

Anal. Calcd. for $C_{24}H_{47}N_5O_9 \cdot H_2O$: C, 50.77; H, 8.70, N, 12.33.
Found: C, 50.35; H, 8.44, N, 12.15.

Lithium aluminum hydride reduction of 6′-*N*-*t*-butoxycarbonylgentamicin C_{1a}.

To a solution of 6′-*N*-*t*-butoxycarbonylgentamicin C_{1a} (0.325 g) in tetrahydrofuran lithium aluminum hydride (0.15 g) was added. The mixture was heated under reflux with stirring and exclusion of moisture for 18 hours. After cooling to 10°C excess hydride was decomposed

by addition of methanol, followed by water. The solution was acidified to pH 2 with hydrochloric acid and solids were removed by filtration through Celite. The filtrate was neutralized by addition of Amberlite IRA-401S resin (OH^- cycle). After removal of the resin by filtration, 20 ml of Amberlite IRC-50 (H^+ cycle) was added and the mixture stirred for 1 hour. The resin was separated, washed with water, and the product eluted with concentrated ammonium hydroxide. After concentration *in vacuo* the product was chromatographed over silica gel (20 g) eluting with a chloroform-methanol-15 N ammonium hydroxide (2:1:0.35) solvent system. Two-ml fractions were collected and monitored by TLC. Fractions 45~49, containing the desired product, were pooled and lyophilized to give gentamicin C_{2b} (0.075 g, 30 %), $[\alpha]_D^{26}+147°$ (*c*, 0.38, H_2O). The pmr, mass spectrum and chromatographic mobility of the product were identical to that of authentic gentamicin C_{2b}.

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